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EPA

Red Algal Sexual Reproduction

Supplemental Report for Training Videotape

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Supplemental Report for the

Red Algal Sexual Reproduction Test

Training Videotape

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Red Algal Sexual Reproduction Test

INTRODUCTION

The U.S. Environmental Protection Agency's (EPA's) Environmental Research Laboratory in Duluth, Minnesota, has developed a series of freshwater toxicity tests to evaluate effluent toxicity. The tests use freshwater fish, invertebrates, and plant species as indicators of toxic effects to lakes, streams, and rivers. The EPA Environmental Research Laboratory in Narragansett, Rhode Island (ERL-N), has adapted the freshwater toxicity testing methods and developed new methods to perform similar testing in the marine or estuarine environments.

This report summarizes methods developed at the Narragansett laboratory for estimating the chronic toxicity of marine or estuarine effluents and receiving waters on the sexual reproduction of the marine macroalga, *Champia parvula*. Males and females are exposed in a static system to effluents or receiving waters for 2 days, followed by a 5- to 7-day recovery period for the female plants in a control medium. Cystocarp production by the female, which indicates sexual reproduction, is used as the endpoint. The methods described in this report and demonstrated in the accompanying tape are detailed in the EPA methods manual, "Short-term Tests for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms" (EPA/600/4-87/028).

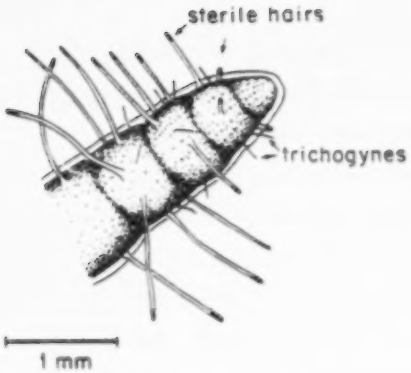
CULTURING *CHAMPIA PARVULA*

Although there are three macroscopic stages in the life history of *Champia*, only the male and female plants are used in toxicity testing. EPA's Environmental Research Laboratory in Narragansett maintains unialgal cultures of *Champia* year round, and plant material is available from the lab for starter cultures.

To keep a constant supply of plant material available, maintain several unialgal stock cultures of males and females simultaneously. New cultures should be started weekly from excised branches so that cultures are available in different stages of development. To guard against microalgal contamination, use sterile technique when culturing the algae - autoclave all stock solutions, and flame all tools before cutting or transferring plants.

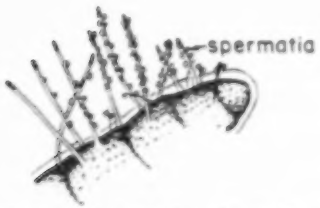
Keep the cultures on a cycle of 16 hours of light and 8 hours of darkness. The light level should not exceed 500 ft-candles. The light may have to be adjusted to that level, depending on the reflecting characteristics of the incubators. The temperature should be maintained at 22 to 24° C and the salinity at 28 to 30 ppt. Natural seawater or a 50-50 mixture of natural and artificial seawaters are best for cultures. Gently aerate the cultures. Change alternate cultures' medium every week, so that if a stock solution should become contaminated, the entire batch will not be lost. While replenishing the medium, divide the growing algae in half with sharp forceps or discard half of the biomass to prevent overcrowding. New cultures can also be started at this time, using 1-cm branch tips. Add nutrients using a pipet, or, as Narragansett has found, a squeeze bottle is quick and easy to use. Recipes for the culturing medium and nutrient solutions are provided in Appendix B.

At the end of approximately 3 weeks, there should be enough plant material to conduct the test. Examine the stock cultures to determine their readiness for testing.



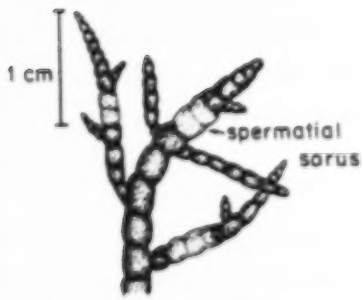
Apical tip of the female branch showing sterile hairs and trichogynes (reproductive hairs) (Aquatic Toxicity Testing Manual, p. 16).

Place a few female branch tips in seawater in a petri dish, and examine them under a compound microscope to determine if trichogynes are present. An inverted scope works best with the petri dishes, although standard slides and microscopes also can be used. Trichogynes are the short, fine reproductive hairs to which the spermatia attach. They should be seen easily near the apex of the branch tip. Although both sterile hairs and trichogynes occur on the apex, sterile hairs occur over the entire plant thallus. Sterile hairs are wider and generally much longer than trichogynes, and appear hollow, except at their tip, where they seem to be plugged.



Spermatia attached to the sterile and reproductive hairs on the female branch (Aquatic Toxicity Testing Manual, p. 17).

Males should be visibly producing spermatia. Sometimes, the presence of spermatial sori can be determined by placing some male tissue in a petri dish and holding it against a dark background. Mature sori can also be easily identified under a microscope along the edge of the thallus. The sorus areas are generally thicker, and lighter in color, than the rest of the plant body. At higher magnification, the spermatia themselves can be seen. The readiness of the male stock culture can also be assessed by placing a portion of a female plant into some of the water from the male culture for a few seconds. Under a microscope, numerous spermatia should be seen attached to the sterile hairs and trichogynes of the female plant.



A portion of a male thallus showing spermatial sori (Aquatic Toxicity Testing Manual, p. 16).

Once readiness is established for both males and females, the test can begin.

CONDUCTING THE TEST

Prepare cuttings from the most healthy-looking plants. Prepare the female cuttings first to minimize the chances of contaminating them with water containing spermatia from the male stock cultures. Place each plant in a petri dish containing a little seawater. Using a fine-point forceps or scalpel, prepare five cuttings from the female plants for each treatment replicate, severing the plant 7 to 10 mm from the ends of the branch. Try to be consistent in the degree of branching in the cuttings, since cystocarps form at the branch tips. For male plants, use one cutting for each treatment replicate, severing the plant about 2 cm from the end of the branch. If there are few branches, or the spermatial sori appear sparse, larger male cuttings may be needed. The cuttings can be kept at room temperature for up to an hour.

The effluent sample should be kept at 4° C in an incubator or refrigerator until use, but should not be stored longer than 24 hours. Under a hood, prepare 5 dilutions using a 0.3 or 0.5 dilution factor, in 300 or 400-ml replicates. At Narragansett, 125-ml Erlenmeyer flasks are used as test chambers, but any clean container can be used. Natural seawater is preferred for the test solutions, although artificial seawater may be used during the exposure part of the test. Maintain the salinity at 28 to 30 ppt, and use colored labels to indicate treatment and replicates.

The 2-day exposure period starts when the algae are added to the test chambers. Add 5 female branches and 1 male branch to each chamber. Pick up the branch at the base or cut end to avoid injuring the tips. Cover the chambers, then expose the cultures to 16 hours of cool white light and 8 hours of darkness a day for the 2-day

exposure and 5- to 7-day recovery periods. Maintain the temperature between 22° and 24° C, and the salinity between 28 and 30 ppt.

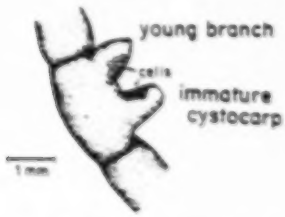
Check on the chambers twice a day, and gently hand-swirl, or shake continuously at 100 rpm on a rotary shaker. Spermatia are not motile, so some motion is critical during the exposure period for reproduction to occur. If desired, the media can be changed after 24 hours. Record the temperature daily from a thermometer placed in a flask of water among the chambers.

After 48 hours, the algae are removed from the effluent samples and allowed to recover for 5 to 7 days. Label recovery bottles with the effluent concentrations tested, and fill with 150 ml of natural seawater and nutrients. Smaller volumes should be be used with a shaker (about half the vessel volume), but make sure that adequate growth will occur without having to change the medium. With forceps, gently remove all of the females from each test chamber, and place them into recovery bottles. When all the replicates have been transferred, place the vessels under cool white light and aerate or shake for the 5- to 7-day recovery period. Aeration will enhance the growth rate of plants in the recovery bottles, although adequate growth will occur using a shaker. Aerate using plastic tubes held in place by foam stoppers.

TERMINATING THE TEST

At the end of the recovery period, drain the chambers and remove the females with forceps, starting with the control plants and ending with those in the

A mature cystocarp. In controls and lower effluent concentrations, they often occur in clusters of 10 or 12 (Short-term Methods manual, p. 286).



A young branch vs. an immature cystocarp (Short-term Methods manual, p. 286).

An aborted cystocarp. A young branch will eventually form at the apex (Short-term Methods manual, p. 286).

highest concentration. Place the female plants between the inverted halves of a petri dish containing a small amount of seawater, and count the cystocarps under a stereomicroscope. Cystocarps are distinguished from young branches by the darkly pigmented spores enclosed in the nodule, and the apical opening for spore release. If there is doubt about the identification of an immature cystocarp, aerate the plants a little longer in the recovery bottles. Within 24 to 48 hours, the suspected cystocarp will either look more like a mature cystocarp or a young branch, or will have changed very little, if at all, indicating an aborted cystocarp. Occasionally cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and, often, by the formation of a new branch at the apex. Dead plants lose their pigmentation and appear white.

FINAL NOTES

The algal sexual reproduction test requires that several criteria be met before the test results are considered acceptable.

- Control plants should average 10 or more cystocarps and there should be no mortality in the control group.

- Control and lowest-concentration exposed algae should be in good physical condition - for example, the branches should not be fragmented. Broken or fragmented branches could indicate that the plants were unhealthy from the beginning of the test.

- The results from the replicate control chambers should be similar.

- All replicates from the affected concentration chambers should show effect.

The methods manual, "Short-term Tests for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms," describes the statistical analysis performed on the test results. The red algal sexual reproduction test is currently used to assess the potential toxic effects of complex chemical mixtures on marine and estuarine organisms. Used in conjunction with chemical-specific methods, this test can provide a comprehensive and effective approach to assessing the impact of complex effluents discharged to the marine and estuarine environments.

REFERENCES

Aquatic Toxicity Testing Seminar Manual. 1985. U.S. EPA Environmental Research Laboratory, Narragansett, RI. ERL-N Contribution No. 796.

(Provides detailed descriptions of the methods used at Narragansett to evaluate the toxicity of discharges to marine and estuarine waters. Served as the basis of a series of seminars conducted by ERL-N personnel.)

Biomonitoring for Control of Toxicity in Effluent Discharges to the Marine Environment. 1989. U.S. EPA Center for Environmental Research Information, Cincinnati, OH; U.S. EPA Environmental Research Laboratory, Narragansett, RI. EPA/625/8-89/015.

(Describes the use of biomonitoring as an effective water quality-based approach to controlling the toxicity of discharges to estuarine and marine waters. Covers regulatory background, testing methods, and case studies.)

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. 1987. Environmental Monitoring and Support Laboratory, Cincinnati, OH. EPA/600/4-87/028.

(Describes methods, quality assurance, laboratory safety, facilities and equipment, data analysis, report preparation, and organism culture and handling for six short-term tests to estimate the chronic toxicity of effluents and receiving waters.)

Technical Support Document for Water Quality-based Toxics Control. 1985. U.S. EPA Office of Water Enforcement and Permits, Washington, D.C.

(Provides guidance for each step in the water quality-based toxics control process, from screening to compliance monitoring.)

APPENDIX A

PREPARING HYPERSALINE BRINE

BACKGROUND

Salinity adjustments are a vital part of using marine and estuarine species for toxicity testing. The majority of industrial and sewage treatment effluents entering marine and estuarine waters contain little or no measurable salts. Therefore, the salinity of these effluents must be adjusted before exposing estuarine or marine plants and animals to the solutions. In addition, it is important to maintain constant salinity across all treatments throughout the test for quality control. Finally, matching the test solutions' salinity to the expected receiving water's salinity may require salinity adjustments. ERL-N uses hypersaline brine, prepared from filtered natural seawater, to adjust exposure solution salinities. Note that commercially available artificial sea salts have not been sufficiently tested, and therefore are not recommended for all of the subchronic toxicity tests at this time.

Hypersaline brine has several advantages over artificial sea salts that make it more suitable for use in toxicity testing. Concentrated brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of test organisms. It may be held for prolonged periods without any apparent degradation. Brine may be added directly to the effluent to increase the salinity, or may be used as control water by diluting to the desired salinity with deionized water. The brine can be made from any high quality, filtered seawater supply through simple heating and aerating.

GENERATING THE BRINE

The ideal container for making brine from natural seawater has a high surface-to-volume ratio, is made of a non-corrosive material, and is easily cleaned. Shallow fiberglass tanks are ideal.

Collect high quality (and preferably high salinity) seawater on an incoming tide to minimize the possibility of contamination. Special care should be used to prevent any toxic materials from coming in contact with the seawater. The water should be filtered to at least 10 um before placing into the brine tank. Thoroughly clean the tank, aeration supply tube, heater, and any other materials that will be in direct contact with the brine before adding seawater to the tank. Use a good quality biodegradable detergent, followed by several thorough deionized-water rinses. Fill the tank with seawater, and slowly increase the temperature to 40° C. If a heater is immersed directly into the seawater, make sure that the heater components will not corrode or leach any substances that would contaminate the brine. A thermostatically controlled heat exchanger made from fiberglass works well.

Aeration prevents temperature stratification and increases the rate of evaporation. Use an oil-free air compressor to prevent contamination. Evaporate the water for several days, checking daily (or more or less often, depending on the volume being generated) to ensure that the salinity does not exceed 100 o/oo and the temperature does not exceed 40° C. If these changes are exceeded, irreversible changes in the brine's properties may occur. One such change noted in original studies at ERL-N was a reduction in the alkalinity of seawater made from brine with salinity greater than 100 o/oo, and a resulting reduction in the animals' general health. Additional seawater may be added to the brine to produce the volume of brine desired.

When the desired volume and salinity of brine is prepared, filter the brine through a 10-um filter and pump or pour it directly into portable containers (5-gallon Cubitainers or polycarbonate water cooler jugs are most suitable). Cap the containers, and record the measured salinity and the date the brine was generated. Store the brine in the dark at room temperature until used.

SALINITY ADJUSTMENTS USING HYPERSALINE BRINE

To calculate the volume of brine (V_b) to add to 0 o/oo sample to produce a solution at a certain salinity (S_f), use this equation:

$$V_b * S_b = S_f * V_f$$

where V_b = volume of brine, ml

S_b = salinity of brine, o/oo

S_f = final salinity, o/oo

V_f = final volume, ml (brine brought to this volume with 0 o/oo sample).

To calculate the volume of brine (V_b) required to raise the salinity of an effluent or receiving water sample (S_s) to a certain salinity (S_f), use this equation:

$$V_b = [(V_f * S_f) - (V_s * S_s)]/(S_b - S_f)$$

where V_b = volume of brine, ml

S_b = salinity of brine, o/oo

V_s = volume of sample, ml

S_s = salinity of sample, o/oo

S_f = final salinity , o/oo

V_f = final volume, ml (final volume is combined brine and deionized water plus the sample volume; percent original sample in the final sample = $V_s/V_f * 100$).

The table on the next page gives volumes needed to make 20 o/oo test solutions from effluent (0 o/oo), deionized water, and 100 o/oo hypersaline brine.

Quantities of effluent, deionized water and a hypersaline brine of 100 o/oo (only) needed for conducting daily renewals of test solutions at 20 o/oo salinity.

Exposure Concentration (%)	Effluent (0 o/oo) (ml)	Deionized Water (ml)	Hypersaline Brine (100 o/oo) (ml)
32	640	960	400
10	200	1,400	400
3.2	65	1,535	400
1.0	20	1,580	400
0.32	7	1,593	400
Control	--	1,600	400

APPENDIX B

NUTRIENTS AND MEDIA

This table lists the additional nutrients to be added to natural seawater for stock cultures and test medium. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 minutes. Adjust the solution to about pH 2 before autoclaving to minimize the possibiity of precipitation.

COMPOUND	AMOUNT/LITER CONCENTRATED STOCK SOLUTION	
	CULTURE MEDIUM	TEST MEDIUM
Nutrient Stock Solution[a]		
$NaNO_3$	6.35 g	1.58 g
$NaH_2PO_4 \cdot H_20$	0.64 g	0.16 g
$Na_2EDTA \cdot 2\ H_2O$	0.133 g	- -
$Na_3C_6H_5O_7$	0.051 g	0.128 g
Iron[b]	9.75 ml	2.4 ml
Vitamins[c]	10.0 ml	2.5 ml

[a]Add 10 ml of appropriate nutrient stock solution per liter of culture or test medium.

[b]Iron stock solution is prepared by dissolving 1 g iron powder in 10 ml concentrated HCl and diluting to 1 liter with deionized water. Acceptable stock solutions could also be made with ferric or ferrous chloride.

[c]Vitamin stock solution: dissolve 2.5 mg B12, 2.5 mg biotin and 4.88 g thiamine·HCl in 500 ml deionized water. Adjust pH to about 4, divide into 10-ml subsamples, and autoclave for 2 min.

APPENDIX C

MATERIALS NEEDED FOR TESTING

Lab Supplies

Fine-point stainless steel forceps

100-ml polypropylene cups with covers (or 125-ml Erlenmeyer flasks) for exposure chambers

Polystyrene petri dishes for holding cuttings and treated plants for counting

100-ml graduated cylinder

1- and 10-ml disposable pipets

Digital micropipets (200 and 1000 ul maxima) if dilutions are made directly in test chambers

Disposable micropipet tips

Recovery bottles or flasks (one per treatment or control chamber)

Aquarium pump(s)

Air tubing (made of non-toxic material)

Plastic aeration tubes (1 ml disposable pipets work fine)

Foam plugs

Thermometer

Marking pens

Colored tape

Data Sheets (Appendix E)

Lab Equipment

Cool-white fluorescent lighting, sufficient to give 75 uE m^{-2} s^{-1} (about 500 ft-candles).

Rotary shaker (hand-swirling the exposure chambers twice a day can be substituted).

Stereo (dissecting) microscope

Refractometer (salinometer)

APPENDIX D

SUMMARY OF TEST CONDITIONS

Test type:	Static, non-renewal
Salinity:	30 o/oo
Temperature:	22 to 24 °C
Photoperiod:	16h light:8h dark
Light source:	cool-white fluorescent
Irradiance:	about 75 uE m^{-2} s^{-1}
Test solution volume:	100 ml
Test chamber size:	110-ml polypropylene cups (with covers) or 125-ml Erlenmeyer flasks
Number of test organisms per test chamber:	5 female and one male branch tip
Number of replicate chambers per treatment:	3
Aeration:	None; chambers are either shaken at 100 rpm on a rotary shaker or hand-swirled twice a day
Dilution water:	30 o/oo natural or artificial seawater with additional nutrients added
Test duration:	2-day exposure followed by a 5- to 7-day recovery period for cystocarp development
Effect measured:	Sexual reproduction (number of cystocarps per female)

APPENDIX E

DATA SHEETS

CHAMPIA PARVULA Cystocarp Data Sheet

Collection Date:
Exposure Began:
Recovery Began:
Counted:

Experiment No.

Treatments: (% Effluent, ug/L, or Receiving Water Sites)

Replicat	Control							
A1								
2								
3								
4								
5								
Mean								
B1								
2								
3								
4								
5								
Mean								
C1								
2								
3								
4								
5								
Mean								
Overall Mean								
St Dev.								
% of Cont								

Temperature: _____
Salinity: _____
Light: _____
Dilution Water: _____

Mean Sq. Error=
Degrees Freedom=
Dunnett's Tab Val=

n cont.=
n treat.=

Critical Valued'=

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